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Rapid Propagation of Virus-free Sugarcane (*Saccharum officinarum*) by Somatic Embryogenesis

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Abstract

Elimination *Sugarcane Mozaic Virus* (*SCMV*) in sugarcane plantlets and propagation via somatic embryogenesis was assessed. The aim of this study was to obtain a virus-free sugarcane and determine methods for propagation via somatic embryogenesis. Explants derived from sugarcane SUT-02 plantlets. The explants were cultured on ribavirin and acyclovir medium at 0, 20 and 40 mgl^{-1} concentrations. Virus-free plantlets detected by DAS-ELISA. Rapid propagation virus-free plantlets through somatic embryogenesis consists of 3 stages. Stage 1: callus induction on the media (a) MSo, (b) MSo + 3 mgl^{-1} 2,4-D, (c) MSo + 4 mgl^{-1} 2,4-D, (d) MSo + 3 mgl^{-1} 2,4-D + 1.5 mgl^{-1} BAP, (e) MSo + 4 mgl^{-1} 2,4-D + 1.5 mgl^{-1} BAP, stage 2: proliferation on media MSo + 1.5 mgl^{-1} 2,4-D and stage 3: regeneration on MSo media. The results showed that the media ribavirin and acyclovir at 20 mgl^{-1} and 40 mgl^{-1} concentration produces 100% virus-free sugarcane plantlets. Best embryogenic callus induction media is MSo + 3 mgl^{-1} 2,4-D and virus free plantlets have produced as much as ± 35 plantlets within 9 weeks.

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Keywords: virus-free sugarcane, Sugarcane Mozaic Virus (SCMV), virus elimination, 2,4-D, Somatic embriogenesis

1. Introduction

Sugarcane (*Saccharum officinarum* L.) is the one of energy source for producing sugar. The cultivation of sugarcane (*S. officinarum* L.) on commercial land often found mosaic disease caused by sugarcane mosaic virus (SCMV). The attack of sugarcane mosaic virus (SCMV) reported can decrease the production of sugarcane around 30-50%. SCMV can be reduced by meristem culture, apical meristem (Naz et al., 2009), somatic embryogenesis (Ramgareeb et al., 2010) and chemotherapeutic. Chemotherapeutic materials are widely used for the treatment of viral diseases which are dithiouracil, ribavirin and acyclovir (Al Maari et al., 2012).

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The quality and quantity of sugarcane needs to be improved by producing healthy seed propagation in large scale using tissue culture techniques through somatic embryogenesis. Propagation via somatic embryogenesis techniques have the potential to be developed. Somatic embryogenesis has several specific stages starting from the formation of pro-embryonic masses (pro-embryonic masses) followed by somatic embryo formation, maturation, and regeneration (Hussein et al., 2006; Purnamaningsih, 2011).

Many factors influence the successful of somatic embryogenesis among other things exogenous auxin, the composition of auxin and cytokine, the source of explants, nitrogen and sucrose. Several studies have reported that the addition of auxin 2,4-D alone increased callus initiation (Ali and Iqbal, 2010). The addition of growth regulators 2,4-D with a concentration of 3-4 mgl-1 can produce sugarcane callus induction the most good when compared with of 2,4-D on the lower concentration 1-1.5 mgl-1 (Ali et al., 2010). BAP widely used for regeneration of sugarcane (Sadat et al., 2011; Ikram and Memon, 2012; Alcantara et al, 2014). The addition of BAP with low concentration can trigger the regeneration of sugarcane (Anjum et al., 2012). The addition of combination of 2,4-D and BAP is the most effective way to induce somatic embryos (Naz et al., 2008; Jahangir and Nasir, 2010).

Explants have been used in previous studies are part of the spindle leaf (Ho and Vasil, 1983; Tiel et al., 2006; Mayang et al., 2011; Anjum et al., 2012). Roy et al., (2011) using a roll of the young sugarcane leaves (spindle leaf) can be used as explants for callus production and induction of somatic embryogenesis. Mustafa and Khan (2012) stated that explants in vitro plantlets from the first segment (basal part) has the best response to induction and callus proliferation. The first segment (basal part) of sugarcane in vitro contains a lot of meristematic cell. The research goal is to get the type of explants and appropriate concentration of antiviral for elimination SCMV and virus-free sugarcane propagation method through somatic embryogenesis.

2. Methods

2.1. Plant Material.

Planting material was sugarcane transgenic plantlets SUT-02 age 1 month derived from laboratory of CDAST Jember University. Plantlets with 2 cm height was used as explants.

2.2. Virus Elimination.

Explants from in-vitro shoots of sugarcane SUT-02 cultured on MS medium supplemented with antiviral A1: acyclovir and A2: ribavirin consisted of K0: 0 mgL-1, K1: 20 mgL-1, K2: 40 mgL-1 concentrations. Explants were eliminated during 6 weeks. Planlet was then sub-cultured on shoot medium consist of MSo + 2 mgL-1 BA + Kinetin 0,5 mgL-1 and rooting medium ½ MS after height shoot up to ± 2 cm.

2.3. DAS-ELISA analysis.

Sample of in vitro leaves were collected 0,2 g, crushed using liquid nitrogen until smooth, then dissolved in SBI buffer by comparison 1 : 3. Elimination of virus-free plantlets was detected by DAS-ELISA Sugarcane Mosaik Virus Kit from AC diagnostics, inc. Data Interpretation: Interpretation was done by measuring absorbance value in 405 nm to ELISA reader. Determining the positive samples of infected plants were done with a cut off value method, determine the mean absorbance value of negative control added by 3 × standard deviation. Percentage of elimination of Sugarcane Mosaic Virus (SCMV) determined as Widianingsih (2009).

2.4. Regeneration by Somatic Embryogenesis.

Plant material for multiplication through somatic embryogenesis using virus-free shoot in-vitro, cut into ± 0,5 cm in the basal of explants. Regeneration through somatic embryogenesis was done trough 3 stages: Callus induction, proliferation, and regeneration. Callus induction medium consist of: (A): MSo, (B): MSo + 3 mgL-1 2,4-D, (C): MSo + 4 mgL-1 2,4-D, (D): MSo + 3 mgL-1 2,4-D + 1.5 mgL-1 BAP, (E): MSo + 4 mgL-1 2,4-D + 1.5 mgL-1 BAP. Explants were incubated in dark condition during 30 days, until embryogenic callus were formed.

Embryogenic callus formed were sub-cultured in proliferation medium: MSo + 1,5 mgL-1 2,4-D + CH 300 mgL-1 + prolin 560 mgL-1 + 30 gL-1 sucrose + 2,5 gL-1 phytagel. Embryogenic callus were incubated for 30 days in the proliferation stage, then sub-cultured to regeneration medium: MSo + 30 gL-1 sukrosa + 2,5 gL-1 phytagel. Callus morphology and somatic embryogenesis stages were observed by binocular microscope.

3. Result and Discussion

Sugarcane varieties that could potentially be developed for the production of sugarcane seedling in large quantity and high quality are the SUT-02. Sugarcane SUT-02 is genetically modified sugarcane overexpression SoSUT1 gene (Sugiharto and Safitri, 2011) which is expected to produce a high and capable of producing high yield. SUT-02 sugarcane plantlets treated with antiviral acyclovir and ribavirin at concentrations of 0, 20 and 40 mgL-1. Analysis of the plantlets that have been treated in trials with was done through DAS-ELISA method. Result of the virus elimination with antiviral was provided on Figure 1.

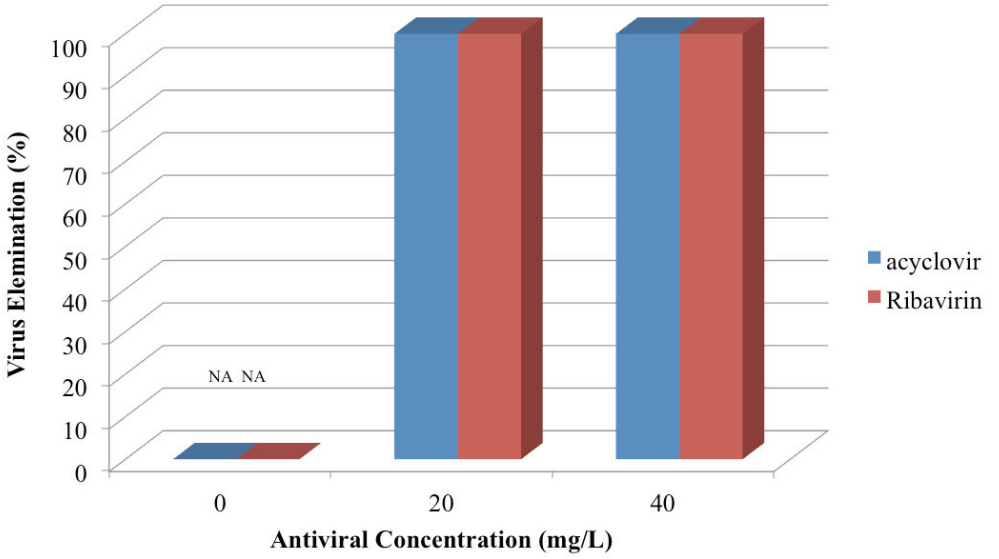

Figure 1. Percentage of virus elimination at different acyclovir and ribavirin concentration based on ELISA test

Results of analysis using DAS-ELISA method showed that the treatment 0 mgL-1 virus particles were not completely eliminated (0%), this indicated that at antiviral with concentrations of 0 mgL-1 still contains the virus particles SCMV. While at antiviral concentrations of 20 and 40 mgL-1 was able to eliminate the virus particles SCMV of 100% (Figure 1). Virus-free sugarcane plantlets can be used to healthy seedling. Propagation via somatic embryogenesis techniques has the potential to be developed. According tp Purnamaningsih (2002) that propagated through somatic embryogenesis has advantages because this technique can produce an unlimited number of propagula in a short time. Explant growth in A media (Figure 2A) did not form a callus but growing shoots and organogenesis occur. Organogenesis is characterized by the growth of shoots from explants. Organ development due to the meristematic activity (meristemoid) leading to the formation of organs, while the addition of growth regulators 2,4-D and BAP stimulates the growth of callus with different characteristics.

Sugarcane SUT-02 explants grown on media induction B with the addition of 3 mgL-1 2,4-D for 3 weeks has formed a callus (Figure 2B). Callus growing from the basal part that has meristematic cells. Likewise, the explants were cultured on induction medium C with the addition of 4 mgL-1 2,4-D (Figure 2.c). Different things shown in explants grown on media induction D and E, combination 2,4-D and BAP (Figure 2.d and 2.e) was not formed the embryogenic callus because callus was browning. Browning occurrence due to the phenolic compounds, so that callus induction and nutrient absorption will be slower (Gill et al., 2004).

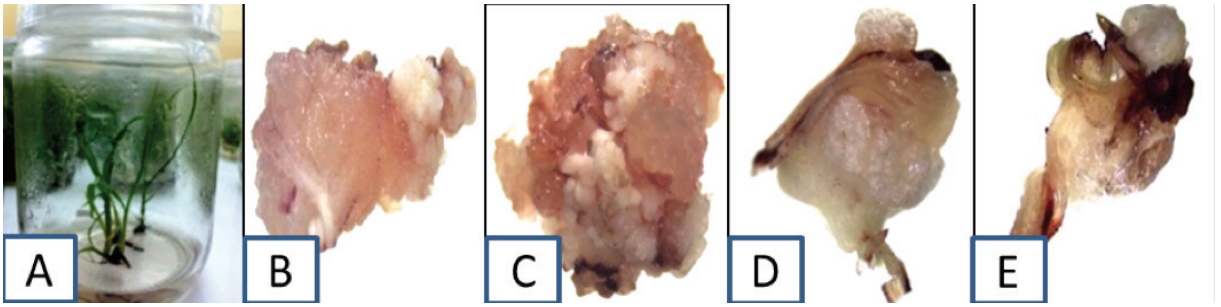

Figure 2. Sugarcane callus morphology in diffrent media. (A) MSo, (B) MSo + 3 mgL-1 2,4-D, (C) MSo + 4 mgL-12,4-D, (D) MSo + 3 mgL-1 2,4-D + 1.5 mgL-1 BAP, (E) MSo + 4 mgL-1 2,4-D + 1.5 mgL-1 BAP.

The addition of 2,4-D showed the best results in inducing callus compared to the addition of 2,4-D and BAP. The addition of 2,4-D and BAP did not produce embryogenic callus, it was similar to that reported by Anjum et al., (2012). Morphological observation on the callus proliferation phase focused to observe the characteristics of embryogenic callus that will differentiate into the stages of somatic embryogenesis. The observation of the morphology of the callus showed characteristics of embryogenic and non-embryogenic callus. Callus morphological differences can be seen in Figure 3.

Formation of somatic embryos produced from the embryogenic callus in proliferation media. The results showed that the non-embryogenic callus with characteristics of compact structure, white or milky white, smooth, non-transparent, tends to blackish brown color. Non-embryogenic callus can not develop into callus and regenerated into plants.

The early stages of embryo formation begins with the formation of mass pro embryo mass (PEM) or the pro globular embryogenic callus which has the structure of a glossy, transparent, and dried (Figure 3A). Pro embryo mass (PEM) will further develop into nodular embryogenic callus-shaped, glossy, crumbs, dry, and transparent to the SUT-02 (Figure 3b).

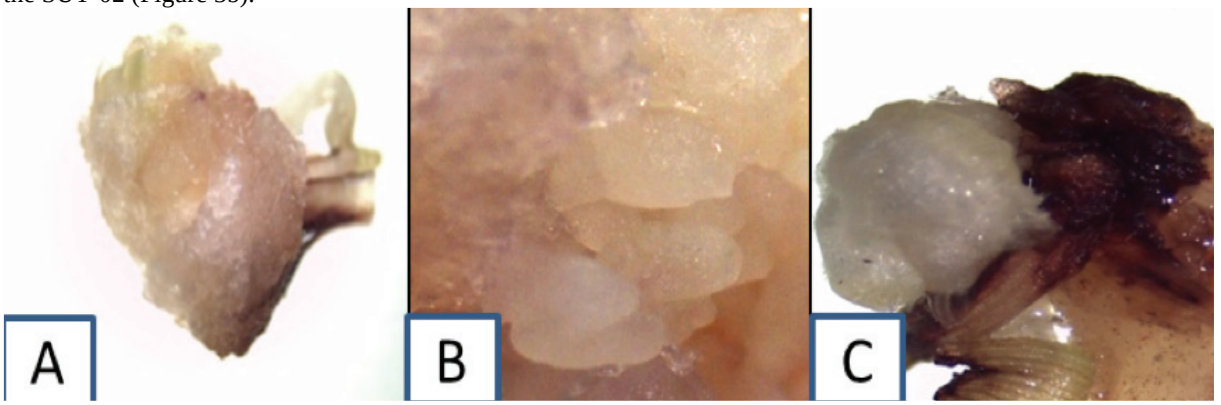

Figure 3. Embryogenic and non-embryogenic callus morphology (A) Pro Embryo Mass (PEM) (B) Embryogenic callus, (C) non embryogenic callus

Observations on callus induction time indicated that the fastest callus formation with addition of 2,4-D were medium B (3 mgL-1 2.4-D) and media C (4 mgL-1 2.4-D). Media D (3 mgL-1 2,4-D + 1.5 mgL-1 BAP) and medium E (4 mgL-1 2,4-D + 1.5 mgL-1 BAP) showed the response of callus formation, but the time taken was more longer than the explants grown on media supplemented with 2,4-D alone (Figure 4.I).

High percentage of callus induction were medium B (3 mgl-1 2,4-D) 100% and medium C (4 mgl-1 2,4-D) 89%. The addition of BAP in different 2,4-D were medium D 11% and medium E 33.3% (Figure 4.II).

Callus at the end phase of the callus induction show that callus in medium B, C, and E can be sub-cultured to the stage of proliferation. Explants on medium A were not sub-cultured in callus proliferation media due to direct organogenesis. While explants in media D does not qualify for callus proliferation stage because the resulting callus did not develop to the next stage.

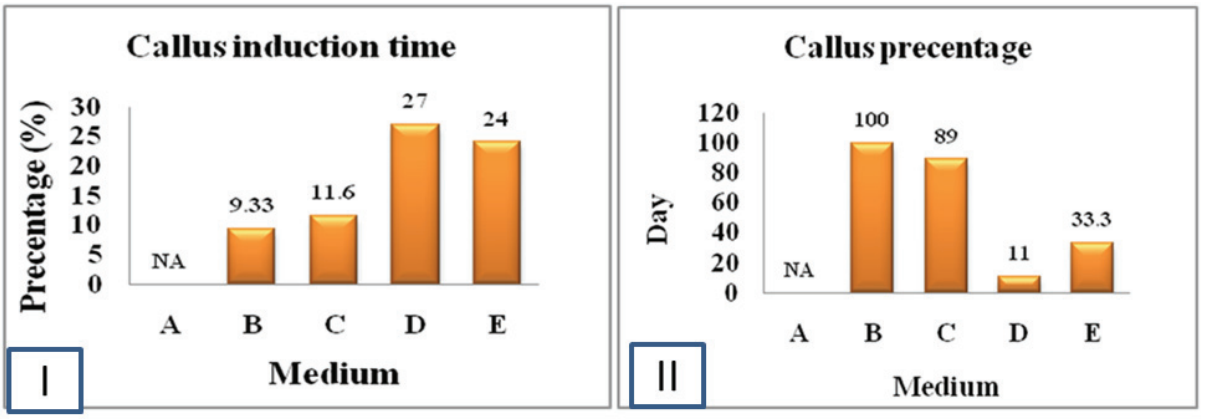

Figure 4. Callus induction time and Callus percentage on different medium (A) MSo,(B) MSo + 2,4-D 3 mgL-1, (C) MSo + 2,4-D 4,5 mgL-1, (D)MSo + 2,4-D 3 mgL-1+ BAP 1,5 mgL-1, (E) MSo + 2,4-D 4,5 mgL-1+ BAP 1,5 mgL-1.

At the time of embryogenic callus proliferation media will trigger the development of pro-embryonic callus structure into zygotic embryos. Stages of somatic embryogenesis in media proliferation were observed after 2 weeks and morphology of the subsequent stages of somatic embryogenesis was observed microscopically to 4 weeks using a stereo microscope. The observation can be seen in Figure 5.

Stages of somatic embryogenesis are globular, scutellum, coleoptile and cotyledons. Stages of somatic embryogenesis were obtained at the time of incubation in media proliferation (Figure 5). Stages of somatic embryogenesis occurred in a short time and its ability decreases as the length of the duration of incubation in medium (Roy et al., 2011).

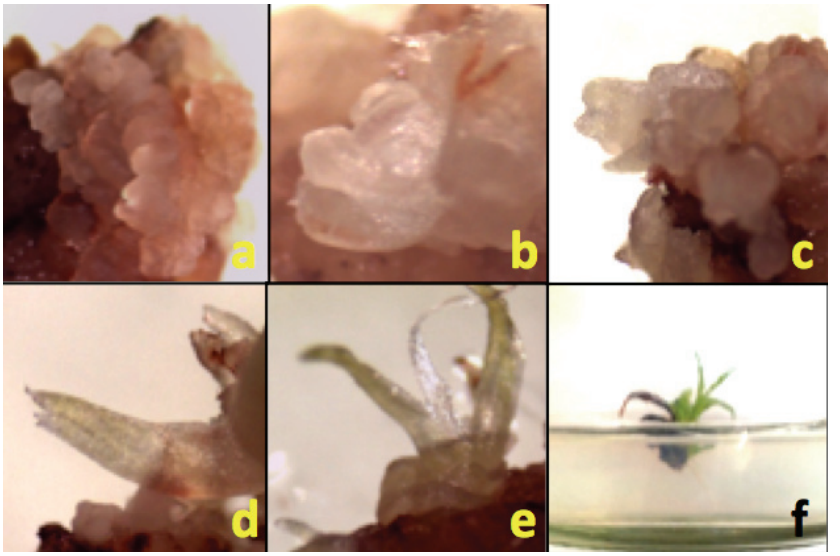

Figure 5. Somatic embryogenesis stage (a) Globular, (b) Scutellar, (c) coleoptile, (d) and (e) cotyledon, (f) shoot.

Coleoptile stage has the potential to serve as an encapsulation material for synthetic seed because it is meristematic. Meristematic nature has the potential to support the development of plant tissue to be complete plants. Stages cotyledons are the result of the development stage of coleoptile obtained at the end stage of maturation. coleoptile will develop into a complete structure with the shoot and root meristem or bipolar (Purnamaningsih, 2002).

4. Conclusion

The results showed that the media ribavirin and acyclovir at 20 mgl^{-1} and 40 mgl^{-1} concentration produces 100% virus-free sugarcane plantlets. In addition, Best embryogenic callus induction media is MSo + 3 mgl^{-1} 2,4-D and virus free plantlets have produced as much as 35 plantlets within 9 weeks

References

Al Maari, K., Massa, R., AlBiski, F., 2012. Evaluation of some therapies and meristem culture to eliminate potato Y potyvirus from infected potato plants. Plant Biotechnology 29, 237-243.

Ali, S., Iqbal, J., 2010. Facile Regeneration Through Adventive / Somatic Embryogenesis from *In Vitro* Cultured Immature Leaf Segments of Elite Varieties of Sugarcane (*Saccharum officinarum* L.). Biologia (Pakistan). 56(1&2), 55-62.

Ali, S., Iqbal, J., Khan, M.S., 2010. Genotype Independent *In Vitro* Regeneration System in Elite Varieties of Sugarcane. Pak. J. Bot. 42(6), 3783-3790.

Alcantara, G.B., Dibax, R., Oliveira, R.D., Filho, J.C.B., Daros, E., 2014. Plant Regeneration and Histological Study of the Somatic Embryogenesis of Sugarcane (*Saccharum* spp). Cultivar RB855156 and RB72454. Acta. Sci. Agron. 36(1), 63-72.

Anjum, N., Ijaz, S., Rana, I.A., Khan, T.M., Khan, I.A., Khan, M.N., Mustafa, G., Joiya, F.A., Iqbal, A., 2012. Establisment of an *In Vitro* Regeneration System as a Milestone for Genetic Transformation of Sugarcane (*Saccharum officinarum* L.) against *Ustiligo scitaminea*. Bioscience Methods 3(2), 7-20.

Gill, N.K., Gill. R., Gosal, S.S. 2004. Factor Enhancing Somatic Embriogenesis and Plant Regeneration in Sugarcane (*Saccharum officinarum* L.*)*. Indian J Biotechnol 3, 119-123.

Hussein, S., Ibrahim, R., Kiong, A.L.P., 2006. Somatic Embryogenesis: An Alternative Method for In Vitro Micropopagation. Iranian Journal of Biotechnology 4(3), 156-161.

Ho, W.J., Vasil, I.K., 1983. Somatic Embryogenesis in Sugarcane (*Saccharum officinarum* L.*)*. The Morphology and Physiology of Callus Formation and the Ontogeny of Somatic Embryos. Protoplasma 118, 169-180.

Ikram-ul-Haq, Memon, S., 2012. Plant Regeneration through Somatic Embryogenesis in Sugarcane (*Saccharum officinarum*. L) cultivar CPF-237. Afr. J. Biotechnol 11(15), 3704-3708.

Jahangir, G.Z., Nasir, I.A., 2010. Various Hormonal Supplementations Active Sugarcane Regeneration *In-Vitro*. J. Agr. Sci. 2(4), 231-237.

Mayang, R.B., Hapsoro, D., Yusnita., 2011. Regenerasi In Vitro Tanaman Tebu (*Saccharum officinarum* L.): Induksi dan Proliferasi Kalus, serta Induksi Tunas. Agrotropika (16)2, 52-56.

Mustafa, G., Khan, M.S., 2012. Reproducible *In vitro* Regeneration System for Purifying Sugarcane Clones. Afr. J. Biotechnol 11(42):9961-9969.

Naz, S., A. Ali., A. Siddique. 2008. Somatic Embryogenesis and Planlet Formation in Different Varieties of Sugarcane (*Saccharum officinarum* L.*)* HSF-243 and HSF-245. Sarhad J. Agric. 24(4), 593-598.

Purnamaningsih, R., 2002. Regenerasi Tanaman melalui Embriogenesis Somatik dan Beberapa Gen yang Mengendalikannya. Agriobio. 5(2), 51-58.

Ramgareeb, S., Snyman, S.J., Van Antwerpen, T., Rutherford, R.S., 2010. Elimination of virus and propagation of disease-free sugarcane (*Saccharum* spp cultivar Nco376) using apikal meristem culture. Plant Cell Tissue Organ Culture 100, 175-181

Roy, M., Hossain, M., Biswas, A., Biswas, M.K., Islam, R., 2011. Plant Regeneration through Somatic Embriogenesis from Leaf Sheath Derived Callus Sugarcane (*Saccharum officinarum* L.*)* var ISD-16. Plant Tissue Cult.&Biotech. 21(2), 143-149.

Sadat, S., Hoveize, M.S., Mojadam, M., Marashi, S.K., 2011. The Study Induction and Regeneration Potential of Sugarcane Varieties SP70-1143 and CP76-331. World Appl. Sci. J. 13(5), 1106-1111.

Sugiarto, B., Safitri H., 2011. A Comparison Studi for *Agrobacterium*-mediated transformation Method in Sugarcane (*Saccharum officinarum* L.*).* Ilmu Dasar. 12(2), 140-141.

Tiel, K., Enriquez, G.A., Fuentes, A.D., Ferreira, A., Coll, Y., Pujol, M., 2006. Development of A System for Rapid Plant Regeneration from In Vitro Sugarcane (*Saccharum officinarum* L.*)* Meristematic Tissue. Biotecnologia Aplicada 23(1), 22-24.